Regulation of the insulin and asialoglycoprotein receptors via cGMP-dependent protein kinase

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De la Vega, Luis A., and Richard J. Stockert. Regulation of the insulin and asialoglycoprotein receptors via cGMP-dependent protein kinase. Am J Physiol Cell Physiol 279: C2037–C2042, 2000.—Biotin regulation of asialoglycoprotein receptor expression and insulin receptor activity has been established in two human hepatoblastoma cell lines, Hep G2 and HuH-7. Second messenger cGMP mimics the effect of biotin on asialoglycoprotein receptor expression at the translational level. Metabolic labeling and subsequent immunoprecipitation indicate that the loss of insulin receptor activity during biotin deprivation was due to suppression of receptor synthesis. Evidence for posttranscriptional regulation of insulin receptor synthesis was provided by rapid biotin induction of receptor synthesis without an increase in gene transcript number. Addition of a cGMP-dependent protein kinase (cGK) inhibitor prevented biotin induction of the insulin and asialoglycoprotein receptors, suggesting that protein phosphorylation propagates the cGMP signal transduction cascade. Coatomer protein COPI was recently identified as the trans-acting factor that regulates in vitro translation of the asialoglycoprotein receptor. Biotin repletion of the culture medium resulted in the hyperphosphorylation of α-COP, which was prevented by simultaneous addition of the cGK inhibitor. These findings suggest that the end point of this cGMP signal cascade is modulated by cGK and that a phosphorylation reaction governs the expression of both receptor proteins.

biotin induction; translational regulation; coatomer protein phosphorylation

Regulated expression of cell surface lectins has been implicated in such diverse processes as endocytosis, bacterial and viral infection, regulation of cell proliferation, homing of lymphocytes, and metastasis of cancer cells (31). The asialoglycoprotein receptor (ASGR) is the hepatocellular prototype of a cell surface lectin that reflects the differentiated state of the liver cell (20, 32, 37). Expression of ASGR was reduced by 60–70%, and the binding of 125I-labeled insulin was reduced by >75% in human hepatoblastoma cell lines Hep G2 or HuH-7 grown to confluence in a minimal essential medium (MEM) supplemented with dialyzed fetal bovine serum (dFBS) (9). In contrast to the dramatic effect on receptor expression, the patterns of 35S-labeled cellular proteins resolved by two-dimensional electrophoresis were remarkably similar, as were the patterns of secreted glycoproteins isolated by solid-phase concanavalin A (10).

Reconstitution of dFBS supplemented medium with a 300- to 350-Da ultrafiltrate of FBS fully restored the expression of ASGR and insulin receptor (IR) (8). The low-molecular-weight factor required for restoration was identified as biotin (33), a water-soluble vitamin that acts as a cofactor of glucose and fatty acid biosynthesis. Although it is not usually considered part of a signal transduction pathway, biotin’s effect on the steady-state expression of ASGR polypeptides could be mimicked in a nonadditive fashion by addition of the second messenger 8-bromo-cGMP (8-Br-cGMP) (33). This suggested that the effect of biotin was mediated through cGMP levels via activation of guanylate cyclase (8, 39). Consistent with this hypothesis were the recent observations that the addition of atrial natriuretic factor or sodium nitroprusside, activators of particulate and soluble guanylate cyclase, resulted in normalization of ASGR biosynthesis (35). Although biotin induction of cGMP was indicated, it was not clear which of the potential cGMP target proteins propagated the cGMP signal transduction cascade: cGMP-dependent phosphodiesterases (activation or inhibition), cGMP-gated ion channels, or cGMP-dependent kinases.

Estimates of the steady-state levels of ASGR subunits H1 and H2 mRNAs indicated that cGMP-regulated expression of the ASGR was at a posttranscriptional level. Resolution of ASGR mRNA on sucrose gradients demonstrated that the addition of cGMP shifts these mRNAs from the ribonucleoprotein fraction into a translationally active membrane-associated polysomal pool (9). These findings suggest that cGMP directly affects the translational regulation of both H1 and H2, as opposed to mediating an alteration in their intracellular processing. Gel shift assays indicated a specific cytoplasmic protein interaction with the 5′-untranslated region (UTR) of the ASGR mRNA (35). Protein purification led to the isolation of a fraction highly enriched in RNA binding activity and the coatomer protein COPI (14), a complex of seven unrelated subunits (30). Northwestern analysis coupled
with peptide sequence identified α-COP as a potential RNA binding protein within this fraction. Antibody-induced RNA supershift confirmed α-COP as the trans-acting factor and indicated that β-COP was also part of the protein-RNA complex (14).

In the present study, we demonstrate that the biotin-dependent loss of cell surface insulin binding was due to a reduction of IR polypeptide synthesis and that, as was the case for ASGR, repletion of the dFBS-supplemented medium with biotin fully restored IR expression. The absence of a significant difference in IR mRNA abundance, regardless of whether cells were maintained in MEM supplemented with FBS, dFBS, or dFBS plus biotin, suggests that this change in IR expression was regulated at a posttranscriptional level. In addition, cGMP-dependent kinase (cGK) was identified as a common downstream element in the biotin-induced signal transduction pathway, regulating both ASGR and IR expression. This, to our knowledge, is the first demonstration that activation of cGK by biotin regulates gene expression at the posttranscriptional level.

EXPERIMENTAL PROCEDURES

Cell culture. The human hepatoblastoma cell line HuH-7, previously shown to require biotin for full expression of ASGR (34), was plated from confluent cultures onto 60-mm dishes (Falcon) in MEM containing 100 mg/dL glucose supplemented with 10% dFBS, 100 μM penicillin, and 100 μg/ml streptomycin. This plating protocol initially deprived all cells of biotin. Medium was changed 24 h after plating to establish the various test conditions. Cells were allowed to grow to near confluence before the application of various experimental protocols.

Western blot. Cells were suspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 30 μg/ml aprotinin from Sigma, A-6279, and 0.1 mM phenylmethylsulfonyl fluoride) and passed 20 times through a 21-gauge needle. After centrifugation at 15,000 g for 10 min at 4°C, the supernatants were used for protein assay and Western blot. Forty micrograms of protein per lane from cell lysates were resolved on 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked for 1 h at room temperature in Tris-buffered saline (TBS)-TWEEN buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.8, and 0.1% Tween 20) containing 10% fat-free milk. The membrane was incubated for 1 h at room temperature in either rabbit anti-human ASGR antibody diluted 1:5,000, rabbit anti-human α- and β-subunit specific IR (Upstate Biotechnology) diluted to 1 μg/ml, mouse anti-human transferrin receptor (TfR) (Zymed) diluted 1:2,000, or rabbit anti-α-COP (kindly provided by Dr. Cordula Harter, Heidelberg University) diluted 1:1,000 in TBS-TWEEN containing 2% fat-free milk and processed for chemiluminescence as previously described (8, 10).

Immunoprecipitation of IR and ASGR. Cells were metabolically labeled with 200 μCi/ml of [35S]Met-Cys (Pro-mix, Amersham) for 15 min in the presence or absence of biotin (10−7 M) for an increasing period of time as previously described (8, 10). Labeled cells were washed with ice-cold 0.05 M PBS, pH 7.4, and harvested by scraping with a rubber policeman into 1 ml of PBS followed by centrifugation at 1,000 g for 5 min at 4°C. The cell pellet was resuspended in lysis buffer (described above) and incubated at 4°C with constant mixing for 30 min followed by centrifugation at 10,000 g for 10 min. ASGR and IR were immunoprecipitated from aliquots of the supernatant containing equal amounts of radiolabeled protein. The protein A/G ultralink (Pierce) recovered immune complex was resolved on a 4–20% gradient SDS-PAGE, the fixed gel was processed for fluorography with Amplify (Amersham Life Science) and, after drying, was exposed to BioMax film with an intensifying screen (Eastman Kodak) at −70°C. The extent of ASGR and IR synthesis during the 15 min of labeling was quantified by densitometric scanning of the exposed film.

Phosphate labeling and immunoprecipitation of COPI. HuH-7 cells were incubated in phosphate-free MEM supplemented with 10% dFBS with or without 10 μM Rp-8(4-chlorophenylthio)-guanosine-3′,5′-cyclic monophosphorothioate, a cGK inhibitor obtained from BioLog for 1 h before labeling. Cells were treated with biotin (10−7 M) or 8-Br-cGMP (1 mM) in the presence or absence of the inhibitor during the 1 h of labeling with 200 μCi/ml [35S]orthophosphate. Anti sera to the α-COPI subunit was added to cell lysates containing equal amounts of 35S-labeled proteins as determined by trichloroacetic acid precipitation. To selectively immunoprecipitate the α-COPI subunit, anti-α-COP (kindly provided by Dr. Cordula Harter, Heidelberg University) was used in high detergent concentration to dissociate the COPI complex (24). Okadaic acid (1 μmol) (Sigma) was added to the lysis buffer. The immunoprecipitated protein was transferred to nitrocellulose and exposed to X-Omat AR film with an intensifying screen (Eastman Kodak) at −70°C.

Northern blot analysis. Samples and gels were prepared according to protocols described previously (42). Hybridization was carried out using Sigma’s PerfectHyb Plus buffer following manufacturer’s instructions. [α-32P]dCTP-labeled probes used pT2IR (human insulin receptor in pTZ19U vector kindly provided by Dr. Paul Pilch, Boston University), ASGR H2b subunit, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RNA abundance was quantified by densitometric scanning of the exposed film.

RESULTS

In conjunction with our previous finding that biotin was required for maximum ASGR expression by Hep G2 and HuH-7 cells, a dramatic reduction of insulin binding to the cell surface was also observed when cells were grown in medium deficient in biotin (i.e., supplemented with dFBS) (34). To determine whether the effect of biotin deprivation directly altered peptide-hormone binding or affected the steady-state level of the IR expression, HuH-7 cells were grown to near confluence in MEM supplemented with FBS, dFBS, or dFBS plus biotin (Fig. 1). Consistent with our earlier observations, Western blot analysis confirmed that ASGR expression was markedly reduced in cells grown in MEM supplemented with dFBS and that addition of biotin (10−7 M) fully restored ASGR expression to the control level. As was the case for ASGR, when cells were grown in medium supplemented with dFBS, the steady-state level of IR expression was markedly reduced, and addition of biotin to the culture medium fully restored IR polypeptide expression to the level exhibited by cells grown in FBS. The reduction in the steady-state level of IR exhibited by cells grown in dFBS was sufficient to account for the 75% loss of cell-surface insulin binding activity previously observed (8).
In contrast to the specific requirement of ASGR and IR, the level of steady-state expression of the TfR was independent of biotin addition to the culture medium (Fig. 1). The lack of a TfR response to biotin was in line with earlier studies that indicated biotin deprivation did not significantly affect either total cellular protein synthesis or the overall pattern of $^{35}$S-labeled proteins resolved by two-dimensional electrophoresis (8, 10). Expression of characteristic TfR by cells grown in medium supplemented with dFBS suggests that biotin deprivation did not induce a global defect in protein glycosylation, which has the potential to reduce IR subunit processing and its ultimate expression at the cell surface (19).

Biotin is not usually considered part of an induction pathway, but its effect on the steady-state expression of ASGR can be mimicked in a nonadditive fashion by the second messenger cGMP and known activators of guanylate cyclase (33), such as atrial natriuretic factor and NO generating sodium nitroprusside (39). To determine whether the effect of biotin deprivation on IR expression can be reverted by cGMP, dFBS-containing media was supplemented with the membrane-permeable cGMP analog (8-Br-cGMP). Consistent with our earlier findings, the addition of 8-Br-cGMP fully substituted for the biotin requirement necessary for ASGR expression (33) (Fig. 1). The maintenance of normal IR expression by addition of 8-Br-cGMP to the culture medium (Fig. 1) suggests that a common or parallel signal transduction pathway exists for the induction of these two receptors.

The rate of biotin induction of ASGR and IR synthesis was determined by immunoprecipitation of metabolically labeled receptor proteins. HuH-7 cells were labeled for 15 min with $^{35}$S-Met-Cys after exposure to biotin for increasing amounts of time (Fig. 2). Due to the short labeling period, only the pro-form of IR resolving at 190 kDa was detectable in the immunoprecipitate. Consistent with the results obtained by immunoblot (Fig. 1), biotin deprivation reduced ASGR and IR synthesis by >80% compared with control cells maintained in medium supplemented with 10% FBS. Within 15 min of biotin addition, the rate of ASGR synthesis reached 87% and IR reached 72% of the FBS control. By 30 min, the rate of ASGR synthesis was equal to that of the control cells, and the rate of IR synthesis was just below the control value (97 and 84%, respectively). No further increase in synthetic rate for either receptor was observed, even 2 h after biotin addition (data not shown). These findings support our original observation that the reduction of specific plasma membrane protein expression due to biotin deprivation was a direct consequence of peptide synthesis inhibition, not accelerated degradation.

To determine whether the induction of IR synthesis in the presence of biotin was reflected by an increase of gene transcript number, the level of IR mRNA was estimated by Northern blot analysis (Fig. 3). No significant difference in the abundance of ASGR (1.35 kb) or the IR (11 and 8.5 kb) transcripts were apparent, regardless of whether cells were maintained in MEM supplemented with FBS, dFBS, or dFBS plus biotin. In contrast to the four IR transcripts (11-kb major and 8.5-, 7.0-, 2.8-kb minor) detectable in Hep G2 cells grown in medium containing a high glucose concentration (450 mg/dl), when Hep G2 cells were grown in a more physiological glucose concentration (100 mg/dl), as is necessary to demonstrate the independent effect of biotin (41), only the major 11-kb transcript was distinctly evident (3). Expressions of IR transcripts by HuH-7 cells appear to respond to glucose concentrations in a like manner. An extended exposure of the Northern blot (2 wk) was required to detect the 8.5-kb IR transcript, and, even after this long exposure, the
7.0- and 2.8-kb transcripts were not evident. The dramatic decrease in the expression of the minor transcripts in response to lower glucose concentrations is consistent with previous findings in a wide variety of cell lines (16). The lack of induction of the minor transcripts points to biotin-regulated IR expression being independent of carbohydrate metabolism.

Induction of ASGR and IR by cGMP could, in principle, be mediated by three different proteins: cGK (17), cGMP-gated ion channels (2), and cGMP-regulated phosphodiesterases (1). To differentiate between the cGMP targets, HuH-7 cells were preincubated with a specific cGK inhibitor, Rp-8-(4-chlorophenylthio)-guanosine-3’,5’-cyclic monophosphorothioate (Rp-cGMPS) (38) before biotin and [35S]Met addition. After 15 min of metabolic labeling, the cells were harvested, and the extent of ASGR and IR synthesis determined by immunoprecipitation. As shown in Fig. 4, the addition of Rp-cGMPS (10 μM) during metabolic labeling inhibited biotin induction of both receptor proteins by over 85%.

Transfection studies with various deletion constructs of the cDNA encoding the ASGR H2b subunit localized the cGMP responsive cis-acting element to a 187-nucleotide fragment of the mRNA 5’-UTR (14, 35). With a gel-shift assay, titration of the 5’-UTR with a cytosolic fraction isolated from HuH-7 cells grown in the presence of Br-cGMP or biotin provided direct evidence for an RNA-binding protein responsive to intracellular levels of cGMP (35). Recently, α-COP, one of the seven unrelated subunits of the coatomer protein COP, was identified as the trans-acting factor bound to the 5’-UTR responsible for in vitro translational regulation of ASGR (14). To assess a potential link between cGK activity and the trans-acting factor, the phosphorylation status of α-COP following biotin or Br-cGMP addition was determined. As illustrated in Fig. 5A, the addition of biotin or Br-cGMP resulted in hyperphosphorylation of α-COP, which was markedly inhibited by the simultaneous addition of the cGK inhibitor Rp-cGMP. To assure that biotin deprivation or treatment with cGK inhibitor had no effect on the expression of α-COP, the steady-state level of the coatomer subunit polypeptide was determined by Western blot analysis. As shown in Fig. 5B, the expression of α-COP was unaffected by the various growth conditions after 24 h of treatment. On the basis of its reported turnover rate (25), had biotin deprivation or Rp-cGMPS inhibited α-COP synthesis, a 50% reduction in expression would have been expected.
DISCUSSION

Biotin is known to regulate the expression of hepatic proteins at both the transcriptional (7, 12) and translational levels (8). ASGR is the hepatocellular prototype of a cell surface glycoprotein that responds to a biotin-initiated signal transduction cascade. In conjunction with our previous finding that biotin was required for maximum ASGR expression by Hep G2 and HuH-7 cells, a dramatic effect on insulin binding to the cell surface was also observed (10). In the present study, we demonstrated that the loss of insulin binding activity as a result of biotin deprivation was due to a reduction in the steady-state level of IR and that addition of the second messenger cGMP prevented this loss (Fig. 1). In contrast to the many effects of biotin on hepatic enzyme expression thought to be secondary to glucokinase induction (26), this appears not to be the case for ASGR and IR due to the rapidity of the response to biotin addition (Fig. 2). The over sixfold induction of IR synthesis in the absence of an increase in gene transcript number (Fig. 3) supports posttranscriptional regulation of IR expression by cGMP. In the case of ASGR, similar findings initiated a line of investigation that ultimately led to the establishment of cGMP via biotin activation of guanylate cyclase as a regulator of translational regulation (14, 30, 35). In the absence of more rigorous proofs, we cannot yet confirm translational regulation of IR synthesis.

To date it has not been resolved which cGMP target protein or proteins mediate the downstream effect of biotin. To differentiate between these cGMP enzyme targets, cells were preincubated with a specific cGK inhibitor before biotin induction and metabolic labeling (Fig. 4). Rp-cGMPS inhibition of ASGR and IR synthesis pointed to cGK as the mediator of the cGMP cascade. Two types of cGK have been identified: cGK I, consisting of α- and β-isoforms (5), and the membrane-associated cGK II (13, 23). Originally thought to be absent from hepatocytes (22), cGK II mRNA has been detected in the Hep G2 cells (29), the hepatoblastoma cell line in which cGMP regulation of ASGR mRNA translation was originally demonstrated (33, 35). Although it is difficult to distinguish between the cGK isoforms, the cGK II isoform has been suggested to be more sensitive than either of the cGK I isoforms to the chlorophenylthio analog of the Rp-cGMPS inhibitor (13, 38). Although when purified from a baculovirus consisting of cade. Two types of cGK have been identified: cGK I, (Fig. 4).

inhibitor before biotin induction and metabolic labeling targets, cells were preincubated with a specific cGK

rp-COP in response to

RP-cGMPS (10 μM) necessary to block biotin induction of IR and ASGR expression compared with the 200 μM needed to substantially inhibit calcium flux in hepatocytes (28), a process usually associated with cGK I (23), suggests that cGK II may be the cGMP target protein.

Unlike previous examples of cGK gene regulation at the transcriptional level (11, 15), our data suggest that a phosphorylation step mediated by cGK regulates IR and ASGR synthesis at a posttranscriptional level. Whether cGK is the end point of the cGMP cascade is still an open question. There may be additional steps yet to be resolved within the cGMP cascade regulating posttranscriptional expression of ASGR and IR. For example, an interrelationship between cGK activity and the Ras/MAP pathway at the level of c-Raf kinase phosphorylation and inhibition of MAP kinase phosphatase 1 expression was demonstrated in baby hamster kidney cells (36). More recently, it has been shown that NO activation of the extracellular signal-regulated kinase (ERK) pathway is dependent on the production of cGMP and that cGK plays a role in the propagation of this signal transduction cascade (18). In addition, cGK has been implicated in the activation of p38 MAPK (4), required for the translational regulation of TNF-α (21). The potential sites of cGK-mediated activation are not limited to direct phosphorylation reactions. cGK has been shown to suppress thrombin-stimulated phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3] production and Ca elevation, two of the most common signal transduction elements in the cell. Resolution of our findings at the biochemical level holds the promise of uncovering the mechanism for discriminatory mRNA translation and the molecular targets for the cGMP signal transduction cascade in liver.

In a previous study, gel-shift analysis using partially purified COPI coatamer suggested that α-COP was the most likely candidate for RNA recognition. However, that result did not exclude the possibility of a coatamer accessory molecule as the primary respondent to intracellular cGMP. Interestingly, PtdIns(3,4,5)P3 has recently been shown to specifically interact with α-COP (6). As a result of the present findings (Fig. 5), we postulate that phosphorylation of α-COP in response to cGMP induction of cGK prevents high-affinity binding of the coatamer complex to the 5’-UTR of the ASGR mRNA, thereby allowing ribosomal scanning to the site of translation initiation. In an analogous reaction, cGK phosphorylation of splicing factor 1 has recently been shown to prevent high-affinity binding to U2AF65 crucial to the formation of the spliceosome with snRNA (40). Unlike ASGR, there is no evidence for α-COP playing a role in IR regulation. Indeed, at this time we have yet to establish the molecular level at which cGK governs IR expression. However, our present findings point to cGK as the physiological mediator of cGMP induction of ASGR and IR and that a phosphorylation reaction governs the expression of both receptor proteins, albeit by potentially different mechanisms.

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REFERENCES


